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Early View

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Methionine supplementation for multi-organ dysfunction in MetRS-related pulmonary alveolar proteinosis

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Take-home message:
Pulmonary alveolar proteinosis related to mutations in *MARS1* is a rare and severe lung disease with early onset and no curative treatment to date. In four affected children we showed that methionine supplementation is an effective treatment.

ABSTRACT

Introduction. Pulmonary alveolar proteinosis related to mutations in the methionine tRNA synthetase (*MARS1*) gene is a severe, early-onset disease that results in death before the age of 2 years in one-third of patients. It is associated with a liver disease, growth failure and systemic inflammation. As methionine supplementation in yeast models restored normal enzymatic activity of the synthetase, we studied the tolerance, safety and efficacy of daily oral methionine supplementation in patients with severe and early disease.

Methods. Four patients received methionine supplementation and were followed for respiratory, hepatic, growth, and inflammation-related outcomes. Their course was compared to those of historical controls. Reactive oxygen species (ROS) production by patient monocytes before and after methionine supplementation was also studied.

Results. Methionine supplementation was associated with respiratory improvement, clearance of the extracellular lipoproteinaceous material, and discontinuation of whole-lung lavage in all patients. The three patients who required oxygen or non-invasive ventilation could be weaned off within 60 days. Liver dysfunction, inflammation, and growth delay also improved or resolved. At a cellular level, methionine supplementation normalized the production of reactive oxygen species by peripheral monocytes.

Conclusion. Methionine supplementation was associated with important improvements in children with pulmonary alveolar proteinosis related to mutations in the *MARS1* gene. This study paves the way for similar strategies for other tRNA synthetase deficiencies.

INTRODUCTION

Pulmonary alveolar proteinosis (PAP) is characterized by alveolar accumulation of lipoproteinaceous material derived from surfactant [1]. It has multiple causes. We previously described a specific type of PAP prevalent on the island of La Réunion, characterized by an early onset, associated liver involvement, systemic inflammation, frequent progression to lung fibrosis, and poor prognosis [2]. Mortality reached 59%. Half of the deaths occurred before the age of two years, despite repetitive and frequent whole-lung lavages (WLL) and other treatments, such as high-dose intravenous (IV) steroids that were used in severely affected patients who displayed important inflammation. Mutations in *MARS1* were subsequently identified as disease causing [3] and the phenotype was referred as Interstitial Lung and Liver Disease (ILLD) in OMIM (#615486). *MARS1* encodes the cytosolic methionine tRNA synthetase (MetRS), which plays a critical role in protein biosynthesis by charging tRNAs with methionine, leading to the formation of methionyl-tRNA. The double homozygous Ala393Thr/Ser567Leu mutations found in the "Réunion" patients are located in the catalytic domain of MetRS and severely impair growth and enzymatic activity in yeast, which is restored by methionine supplementation [3]. Enzymatic preparations purified from transfected *E. coli* have confirmed the significant impact of the mutations on the rate of the aminoacylation reaction (reduction of the k_{cat} by 5 to 6-fold relative to wild type), especially for methionine affinity, as shown by a significant increase in the K_m for methionine in mutants [4]. Patients of other ethnicities have been described, with other mutations but a very similar phenotype [3, 5–10]. As enzymatic activity can be restored by methionine supplementation in yeast, we aimed to treat successive patients with standardized methionine supplementation to assess safety and tolerance of such supplementation, and to compare the evolution of the treated patients to that of our historical cohort.

MATERIAL AND METHODS

STUDY DESIGN

This study was approved by the Comité de Protection des Personnes Est-II (CPP18/11/28/42028) and registered at clinicaltrials.gov (NCT03887169). It aimed to determine the efficacy, safety and tolerance of daily oral supplementation of methionine in patients presenting PAP due to the Ala393Thr/Ser567Leu mutations in *MARS1*. Patients were referred to the Paediatric Pulmonology Unit at Necker hospital for their care. Written informed consent was obtained from the parents. Outcomes were assessed at 2 months of treatment. The supplementation was pursued if it was efficient and well tolerated. Data are presented at 3 time-point assessments: before starting the supplementation (D0), 2 months after the supplementation was started (M2) and at last follow-up (LFU). In France, methionine has the status of a nutritional supplement and is widely and freely available.

ADMINISTRATION SCHEME

Methionine was given as L-methionine powder diluted in water and was administrated orally or enterally, every 6h, starting at 80 mg/kg/day and progressively increased until obtaining plasma concentrations between 45 and 500 µM at residual and peak states (1 h after intake). Methods to determine the doses, targeted ranges and frequency of supplementation are detailed in the Online Supplement.

MEASURED OUTCOMES

Efficacy of the treatment was evaluated based on the respiratory, hepatic, inflammatory, and growth status. Respiratory assessment included regular clinical evaluation, chest CT at D0, M2 and at LFU, pathological aspects of broncho-alveolar lavage (BAL) fluid, and the possibility to discontinue the WLL. Liver status was assessed by clinical examination, liver ultrasound, and

liver function tests. Growth and nutritional status were assessed by monitoring growth curves and albuminemia. Systemic inflammation was assessed by measuring CRP, the erythrocyte sedimentation rate, blood neutrophils count and IgG levels.

Patients were monitored for potential adverse effects that included liver dysfunction and central nervous system (CNS) abnormalities, especially a risk of cerebral oedema as described in congenital hypermethioninemia when methionine plasma level exceeds 800 µM [11]. Other possible adverse events were variations in arterial blood pressure, nausea, vomiting, dizziness and polyuria as described in subjects receiving a loading dose of methionine to study the relationship between homocysteine levels and cardiovascular disease. Those effects were mild and transient [12]. Plasma concentrations of homocysteine, which derived from methionine, were monitored. Supplementation with vitamins B6, B9, and B12 was initiated when homocysteine exceeded 30 µM to favour the remethylation of homocysteine back to methionine [13].

COMPARISON TO THE HISTORICAL CONTROLS

We compared the course of treated patients to that of patients reported by Enaud *et al.*[2] as well as seven patients who were diagnosed since this publication. All those patients harboured the Ala393Thr/Ser567Leu genotype.

WESTERN BLOT

MetRS protein expression in PBMCs was assessed by Western blot. Methods are detailed in the Online Supplement.

PRIMING OF ROS PRODUCTION BY PERIPHERAL MONOCYTES

Function of peripheral monocytes was assessed by quantifying reactive oxygen species (ROS) production as detailed in the Online Supplement.

STATISTICAL ANALYSES

For historical controls (HC), data were expressed as median and interquartile range. We computed the difference between the values for each continuous variable at D0 and at LFU for each treated patient, as well as between diagnosis and six months to one year from diagnosis for the HC. Differences were compared between groups using Mann-Whitney tests. For each categorical variable (i.e., weaning from oxygen and enteral nutrition), we compared the proportion of patients who were weaned from such support at the second assessment between groups using Fisher's exact test. A $p\text{-value}<0.05$ was considered statistically significant.

RESULTS

DISEASE COURSE AND EFFICACY OF METHIONINE SUPPLEMENTATION

The patients' characteristics before treatment are presented in Table 1 and disease course for each patient is summarized in Table 2.

Patient 1

P1 was referred to her local hospital at 4 months of age for vomiting, failure to thrive, enlarged liver, and tachypnoea. At that time, chest CT showed only discrete lesions (Figure 1). However, BAL fluid was macroscopically opalescent and its pathological examination confirmed the diagnosis of PAP (Figure 1). Molecular diagnosis of *MARS1* mutations was subsequently made. Before starting methionine supplementation, P1 had severe growth failure, hypotonia, required continuous supplemental oxygen, enteral nutrition and experienced chronic vomiting. Laboratory parameters showed anaemia, cholestasis, mild elevated AST, hypoalbuminemia,

inflammation and high IgG level (Table 2). Ultrasound showed hepatomegaly with hyperechoic parenchyma. Brain MRI was normal. Supplementation with methionine was started at six months of age. She underwent seven therapeutic WLL from D7 to D61 of treatment. She was weaned from oxygen on D42 and enteral nutrition on D54, with resolution of vomiting. On M2, all clinical and biological features were improved (Table 2). Chest CT showed improvement. Echogenicity of the liver normalized. We decided to pursue the treatment. She was discharged home on D71. She was admitted for a new assessment at nine months of age, one month after the last WLL. The BAL showed improvement with total clearance of the extracellular lipoproteinaceous material and a marked decrease in the proportion of vacuolized ORO^+ macrophages (Figure 1). At the last follow-up ten months later, she was asymptomatic. Her weight had reached the mean on the growth curve (Table 2). There was no neurological impairment neither developmental delay. She was not taking any other treatment apart from methionine (30 mg/kg/6h) and no therapeutic WLL had been performed since D61. Treatment every 6h led to reproducible residual and peak values of methionine plasma concentrations (Supplemental Figure S1). Apart from a moderately persistent elevated sedimentation rate, all her laboratory parameters returned to normal (Table 2). Size and echogenicity of the liver normalized. Her chest CT showed very discrete postero-basal ground glass opacities, with no signs of fibrosis (Figure 1).

Patient 2

In P2 *MARS1* related PAP was diagnosed at 5 months of age. She had already undergone 25 WLL. She received monthly IV steroid pulses (300 mg/m²/day for 3 days each) and daily oral steroids from the age of 11 months in order to control her respiratory and inflammatory status. She became steroid-dependent with respiratory relapses when decreasing steroids and developed several complications with systemic arterial hypertension and osteoporotic fractures.

She was started on mycophenolate mofetil (MMF) at the age of 21 months, which allowed tapering then stopping the steroids at the age of 25 months, and spacing the WLL every six months. She was the first patient treated with MMF. She still showed feeding difficulties, refusing oral feeding, presenting regular vomiting, and requiring total enteral nutrition using a gastrostomy. At the time by which methionine supplementation was started, she had WLL every 6 months, and had been taking MMF for 15 months. She displayed discrete persistent inflammation (slightly elevated values of the sedimentation rate and IgG) that resolved at the last follow-up (Table 2). She underwent one WLL that showed only mild lipoproteinaceous material deposition. After 2 months of supplementation with methionine, she was starting to eat by herself and nausea and vomiting disappeared. We decided to pursue the supplementation. After one year of treatment (last follow-up), she showed a significant decrease in her feeding difficulties, along with satisfactory growth (Table 2). Her chest CT, which was already greatly improved after MMF initiation, showed no further changes after methionine supplementation. There were no signs of fibrosis. WLL and MMF were discontinued. She was taking methionine at a dosage of 27 mg/kg/6h, with reproducible residual and peak values of methionine plasma concentrations (Supplemental Figure S1).

Patient 3

At diagnosis at 4 months of age, and before starting methionine supplementation, P3 displayed a similar clinical, biological, and pathological presentation as P2 (Table 2 and Figure 1). Her chest CT showed a more pronounced crazy-paving aspect (Figure 1). Ultrasound showed an enlarged liver. Brain MRI showed periventricular cysts. Supplementation with methionine was started at six months of age. She underwent two therapeutic WLL on D16 and D45. Vomiting decreased from D10 and finally ceased on D31. She was weaned from oxygen on D47 and enteral nutrition on D71. On M2, all clinical and biological features were improved (Table 2).

Control chest CT showed a clear improvement, with regression of posterior consolidations and only persistent scattered subpleural pseudonodular consolidative lesions. The size of the liver decreased on ultrasound. Because of these results, we decided to pursue the treatment. She was discharged home at M2. She was admitted for a new assessment at 11 months old. Clinically, her respiratory and growth status continued to improve. Her chest CT showed new improvement, with almost complete regression of the subpleural pseudonodular consolidations. A subtle very low-density crazy-paving pattern remained, with no signs of fibrosis. She underwent a BAL, which showed partial regression of the extracellular abnormal lipoproteinaceous material and a marked decrease in the number of vacuolized ORO^{+} macrophages (Figure 1). Size of the liver normalized on ultrasound. At the last follow-up 5 months later, she had been taking methionine for 10 months with no other treatment. Her current dosage was 28 mg/kg/6h with stable methionine plasma concentrations (Supplemental Figure S1). She had no respiratory symptoms and growth status continued to improve (Table 2). She had no neurological impairment nor developmental delay and brain MRI was not controlled. Her chest CT showed new improvement with only discrete lesions (Figure 1).

Patient 4

In P4 *MARS1* related PAP was diagnosed at 3 months of age. Before starting methionine, despite repetitive WLL (n=19) and monthly IV steroid pulses, P4 was severely affected by chronic respiratory insufficiency, requiring continuous non-invasive ventilation (NIV) with oxygen, and growth failure and recurrent vomiting necessitating exclusive parenteral nutrition (Table 2). He had a severe psychomotor delay, was not able to sit without support and vocalized a few syllables. Laboratory parameters showed anaemia, cholestasis, hypoalbuminemia, inflammation and high IgG level. He was dependent on blood transfusions and albumin perfusions. Liver was enlarged and hyperechoic on ultrasound. Chest CT showed a crazy-

paving appearance, with an increasing posterior and inferior gradient of density, along with microcystic lesions suggestive of early-stage fibrosis (Figure 1). Brain MRI was normal on two occasions. The last WLL and the last IV steroids pulses had been performed one month before the beginning of methionine supplementation. After starting methionine at 21 months of age, he was weaned from NIV on D38, with a progressive decrease in oxygen supply. He was weaned from parenteral nutrition on D87. The last blood transfusion and albumin perfusion were performed on D79 and D56, respectively. A chest CT performed after two months of treatment showed a marked decrease in the density and extension of consolidations, microcystic lesions remained stable. On ultrasound, size of the liver was stable but echogenicity returned to normal. The patient has not undergone therapeutic WLL nor received steroids or other treatment since the beginning of methionine supplementation. At the last follow-up at 33 months of age, he was taking methionine at a dosage of 20 mg/kg/6h with stable plasma concentrations (Supplemental Figure S1). There was a marked catch-up in growth, his anaemia and cholestasis had resolved, the albumin plasma levels had improved. He was completely weaned off oxygen. He has started to eat by himself. There was also a catch-up in psychomotor milestones as he had a vocabulary of more than ten words, understand and followed simple directions, and walks. Chest CT showed further improvement with an important regression of consolidations, microcystic lesions remained stable.

COMPARISON TO THE HISTORICAL CONTROLS

The cohort of HC included 41 patients. Twenty-five (61%) died at a median age of 3.5 years [1.1-16.5] from terminal respiratory failure. We compared the course of treated patients from D0 (“M0”) to LFU (“M6-M12”) to that of the HC from diagnosis (“M0”) to six months to one year of progression of the disease or at the last evaluation if they died within six months of diagnosis (“M6-M12”). First time point considered for the HC was diagnosis because it

corresponds to the beginning of WLL that used to be the standard of care for those patients. Thus, for patients treated with methionine and for the HC, initial data correspond to status before starting treatment. Among the HC, data were available for comparison at the two assessment points for 20 (11 of which are dead). Their characteristics at diagnosis were similar to those of the patients treated with methionine. The median age at diagnosis was four months [3.3-6.8]. During the course of their disease, they were treated by repetitive WLL alone (n=9/20) or by WLL and systemic steroids (n=11/20). The median number of therapeutic WLL from diagnosis to the second assessment was 13 [10-19]. No patient received MMF. At diagnosis, 15 patients required supplemental oxygen and 18 enteral nutrition. Before starting methionine, 3 patients required oxygen and 4 an enteral or parenteral nutrition. At the second assessment, 1/15 had been weaned off oxygen and none off enteral nutrition, versus 3/3 patients treated with methionine for oxygen and 2/4 for enteral nutrition (p=0.005 for oxygen weaning, p=0.026 for enteral nutrition weaning). Among the five HC who did not initially required oxygen, two worsened and required oxygen at the second assessment. Among the two HC who did not initially require enteral nutrition, one required nutritional support at the second assessment. In HC, repetitive WLL and steroids did not lead to significant improvement in chest CT images and BAL fluid composition as illustrated in Figure 2 for two patients. The patient presented in Figure 2A was diagnosed at 5 months of age. Chest CT and BAL are presented at diagnosis and six months later after 9 WLL. By that time, he was dependent on oxygen and NIV. He is currently 8.5 years old and still required oxygen and nocturnal NIV. The patient presented in Figure 2B was diagnosed at 3 months of age. Chest CT and BAL are presented 12 months later after 18 WLL and 7 series of IV steroid pulses. By that time, he was dependent on oxygen. He died one month later. Regarding other clinical and biological features, differences between values at M0 and M6-M12 were statistically significant between HC and treated patients for respiratory rate (p=0.025), blood neutrophils (p=0.034), AST (p=0.004) and GGT

(p=0.038) (Supplemental Figure S2), showing a greater improvement of these parameters for patients treated with methionine than for the HC. These results suggest efficacy of methionine not only in improving the respiratory status but also inflammation, nutrition, and liver status.

SAFETY OF THE TREATMENT

Methionine supplementation was well tolerated during the protocol and after. Nausea and vomiting occurred in the four patients but pre-existed the treatment and stopped after initiation of it. P3 presented initially mild elevated transaminases (Tables 1 and 2), which normalized on D5 of treatment. On D21 she presented a new episode of elevated transaminases (>3N), and a reappearance of vomiting, feeding difficulties and weight loss. Infectious work-up was negative. These alterations were associated to a parallel rapid decrease in methionine plasma concentration probably explained by a rapid weight gain (+500 g between D1 and D21 of treatment, i.e., a 12.5% increase in the patient's weight). As liver failure with elevated transaminases is itself one of the features of *MARS1* related PAP, the observed decrease in methionine plasma level was hypothesized to be actually the cause of these alterations. A complementary analysis of the literature found data supporting this hypothesis: in animal models, methionine restriction induces steatohepatitis [14–16]. Methionine doses were increased and resulted in an increase in methionine plasma levels, along with a rapid improvement in AST and ALT values, resolution of vomiting, resumption of oral feeding, and weight gain. No recurrence of elevated transaminases has occurred since. Plasma homocysteine never reached the threshold of 30 µM for any of the patients.

CELLULAR ASSAYS

MetRS protein levels in PBMCs of P1 before starting methionine were normal relative to those of a control individual (Supplemental Figure S2). GM-CSF priming of ROS production by

peripheral monocytes was measured in P1 and P3. It was low at baseline and improved after 3 months of supplementation with methionine (Supplemental Figure S2).

DISCUSSION

We report the results of supplementation with methionine for PAP and multi-organ dysfunction caused by hereditary MetRS deficiency. The treatment led to an important improvement in clinical, laboratory, imaging, and pathological parameters and was well tolerated. Peripheral monocytes showed an initially altered function that improved under treatment.

Drawing on our preliminary results [3], two other groups reported methionine use in patients harbouring other *MARS1* mutations. In the case reported by Rips et al., methionine supplementation was briefly mentioned as leading to a clinical improvement but no details on the administration modality, doses, plasma concentrations, or potential cellular assays were provided [17]. Lenz et al. recently published a report on two brothers [18]. Although they provide some data on the doses and plasma concentrations of methionine, they are insufficient to assume that methionine plasma levels were within the target range during 24-hour periods or for days or months of treatment. The index case simultaneously received methionine and other treatments (IV immunoglobulins, hydroxychloroquine, steroids and antibiotics). No chest CT nor BAL examination comparing before and after treatment is given. His older brother was diagnosed during familial screening but was pauci-symptomatic. The efficacy of methionine supplementation is thus difficult to determine from these articles. The publication of isolated case reports reflects the difficulty of drawing powerful conclusions for therapeutic innovations in this orphan disease.

Almost all patients harbouring the “Réunion” mutations have been followed in our reference centre, allowing precise comparison of the treated patients to historical controls. Collected data were compared to patients that had had the standard of care for this disease, including repetitive

WLL. Such a comparison showed significant differences in the evolution of respiratory, nutritional, liver and inflammatory status. P1 and P3 are the first to attain complete respiratory remission at their age. The relapse of hepatic and digestive signs in P3 when methionine plasma concentration decreased and the disappearance of those symptoms once plasma concentrations reached the target range again also argue for the accountability of methionine supplementation on the favourable outcome in the treated patients.

The small sample size and the heterogeneity of the four patients are limitations of this study. *MARS1* related PAP is a rare disease with an incidence of 1 in 10,000 new-borns in Réunion and nearby, and only a few case-reports have described this disease in other ethnic groups [3, 5–10], explaining the small sample size. P2 and P4 did not achieve complete remission of their liver involvement at the last follow-up with a persistent enlarged liver. Treatment was started later than for P1 and P3. This can explain, in part, the discrepancy in their responses to treatment. A prospective follow-up of these patients and the treatment of other children will make it possible to determine the best scheme for treatment and predict its efficacy at various stages of the disease. The results obtained for P1 and P3 argue for starting methionine supplementation as soon as possible.

One strength of this study is the correction of the phenotype at the cellular level by the methionine supplementation. As for other forms of PAP, *MARS1* mutations are hypothesized to induce alveolar macrophage dysfunction, leading to altered pulmonary surfactant metabolism. However, this has not yet been proven. We thus quantified the oxidative burst by the peripheral blood monocytes from two patients, as this would provide indirect information on alveolar macrophage function. Priming of ROS production by monocytes was very low before treatment and improved after three months of treatment. These results are the first to suggest macrophage dysfunction subsequent to *MARS1* mutations and to show such an improvement after treatment.

Tolerance of the treatment was good, consistent with studies on congenital hypermethioninemia, which did not reveal toxicity at the plasma methionine concentrations observed in our study [11, 19]. Oral intake at fixed intervals resulted in stable blood concentrations within each patient. In the era of personalized medicine, which promotes the development of costly and highly specific targeted drugs, L-methionine is highly affordable. WLL, which remains currently the standard of care for these patients, is a heavy and risky procedure performed under general anaesthesia and requires repetitive hospitalizations. In addition, this treatment has never enabled complete remission in the past and appears to have no influence on the long-term prognosis [2]. Thus, methionine appears to be a highly promising and cost-effective treatment.

This study paves the way for similar strategies for other ARS deficiencies. Fuchs et al. reviewed 112 patients with diverse ARS deficiencies that share common features, including lung and liver disease and failure to thrive [20]. The authors proposed a pathophysiological model in which disorders result from insufficient aminoacylation activity to meet translational demand in specific organs. They already suggested supplying the corresponding amino acid for each specific ARS deficiency as a therapeutic approach. Our results provide a first proof of concept for this strategy.

In conclusion, oral methionine supplementation in four children with PAP and multisystemic dysfunction related to *MARS1* mutations led to full remission of the disease in two patients and a clear ongoing improvement in the two others. These promising results will fundamentally change the prognosis of this severe and often fatal disease. They also offer promising therapeutic perspectives for similar strategies in other ARS deficiencies.

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AUTHORS CONTRIBUTIONS

AH, DD, CPo, MMHN, JEB, MS, TJM, LB, CO, JMT, JdB and CD contributed to the conception and design of the study, the acquisition, analysis and interpretation of data for the study.
LA, EG, CPe, CV, FL and SR contributed to the acquisition of the data for the study and revised the draft of the manuscript.
AH, DD, and CD drafted the work and revising it after revision by the other authors.
All authors approved the final version to be published.

CONFLICT OF INTEREST

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A Hadchouel has a patent EP 21 305 689.8 pending.

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Table 1. Patient characteristics at baseline

	P1	P2	P3	P4
Ethnic origin	Réunion	Mayotte	Réunion	Comoros
Gender	Girl	Girl	Girl	Boy
Age at diagnosis (months)	4	5	3	3
Age at treatment's initiation (months)	6	35	6	21
Respiratory status				
Oxygen dependency	Yes	No	Yes	Yes
Ventilatory support	No	No	No	Yes
Growth and nutrition				
Weight (SD)	4.2 (-3.4)	14.9 (+1)	4.1 (-3.7)	8.4 (-2.7)
Regimen	Enteral (65%) + Oral (35%)	Complete enteral nutrition	Enteral (70%) + Oral (30%)	Complete parenteral nutrition
Vomiting	Yes	Yes	Yes	Yes
Neurodevelopmental assessment				
Clinical impairment	Yes, hypotonia	No	Yes, hypotonia	Yes, sat with support, vocalizes syllables, no words
Brain MRI*	Normal	Periventricular cysts	Periventricular cysts	Normal
Liver status				
Enlarged liver	Yes	Yes	Yes	Yes
Elevated AST/ALT	Yes	No	Yes	No
Elevated GGT	Yes	No	Yes	Yes
Haematological status				
Anaemia	Yes	No	Yes	Yes
High neutrophils' count	Yes	No	Yes	Yes
Thrombocytosis	Yes	No	No	No
Inflammation‡	Yes	Yes	Yes	Yes
Daily methionine intake at baseline (mg)	231	650	256	297
Fasting methionine plasma concentration (μM)†	18	33	13	23

P: patient. SD: standard deviation. *: brain MRI was performed at diagnosis for P1, P2 and P3, and at 6 and 22 months old for P4. ‡Inflammation was diagnosed by the presence of a high level of CRP, a high sedimentation rate, or both. †: normal laboratory values: 17-45 μM.

Table 2: Disease course under methionine supplementation

	P1			P2			P3			P4		
	D0 6 months	M2	LFU 19 months	D0 3 years	M2	LFU 4.5 years	D0 6 months	M2	LFU 16 months	D0 21 months	M2	LFU 33 months
Clinical features												
Weight (kg) (SD)	4.2 (-3.4)	4.9 (-3.3)	10.2 (0)	14.9 (+1)	15 (+1)	17.3 (+1)	4 (-3.7)	5.3 (-3)	8.4 (-1.6)	8.4 (-2.7)	10 (-1.5)	10.6 (-2)
Respiratory rate (cycles/min)	70	32	32	28	24	18	60	55	27	65	65	42
Oxygen dependency	1 L/min	No	No	No	No	No	0.5 L/min	No	No	2-4L/min + c-NIV	D: 0.5 L/min N: 0.8 L/min	No
Nutritional regimen	65% E 35% O	100% O	100% O	100% E	80% E 20% O	35% E 65% O	70% E 30% O	35% E 65% O	100% O	100% P	80% P 20% E	70% E 30% O
Nausea / vomiting	Yes	No	No	Yes	No	No	Yes	No	No	Yes	No	No
Psychomotor impairment	Hypotonia	None	None	None	None	None	Hypotonia	None	None	Important delay	Sat alone, said a few words	Walks
Biological features												
Haemoglobin (g/dL)	9.1	10.4	11.7	11	11.3	11	8.7	10.1	10.8	9.2	9.3	11.5
Leucocytes (/mm^3)	31,700	5,100	6,900	6,400	9,200	8,300	20,900	13,500	15,700	18,820	8,200	7,700
Neutrophils (/mm^3)	18,100	1,900	1,400	1,700	4,500	2,400	11,200	3,100	3,300	14,490	3,100	2,200
Platelets (/mm^3)	668,000	218,000	339,000	157,000	235,000	252,000	441,000	565,000	406,000	189,000	183,000	165,000
Sedimentation rate (mm)	56	46	25	11	7	7	87	43	58	34	27	44
CRP (mg/L)	53.8	8.3	<5	<5	<5	<5	15.8	<5	<5	71.4	<5	<5
IgG (g/L)	21.52	10.94	8.03	9.23	10.89	8.68	18.03	11.3	13.93	15.07	11.32	13.34
Albumin (g/L)	23.2	38.4	42.3	38	44.2	37.1	28.7	43	45.2	6	26.5	30
Prothrombin rate (%)	54	71	86	91	85	92	72	85	82	81	71	80
AST (IU/L)	81	44	52	45	28	31	101	51	48	50	108	57
ALT (IU/L)	23	16	25	28	22	23	65	29	22	7	44	24
GGT (IU/L)	64	44	13	16	17	12	406	18	16	198	214	71
T bilirubin (µM)	47	4	4	4	4	4	4	3	2	30	13	4
C bilirubin (µM)	36	2	0	0	0	0	4	0	0	13	0	0
Other treatments												
WLL	+	-	-	+	-	-	+	-	-	+	-	-
Steroids	-	-	-	-	-	-	-	-	-	+	-	-
MMF	-	-	-	+	+	-	-	-	-	-	-	-

SD: standard deviation. D: day. N: night. NIV: non-invasive ventilation. M2: month 2. LFU: last follow-up.

FIGURE LEGENDS:

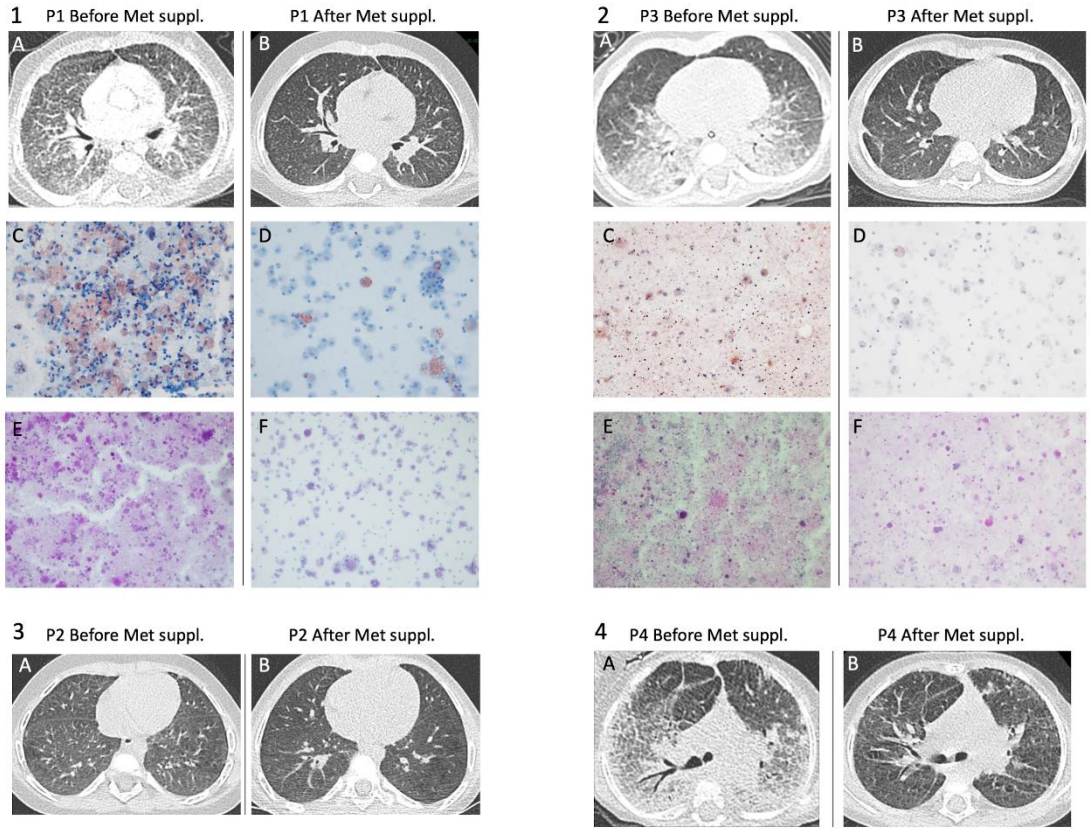


Figure 1. Comparison of imaging and pathological data before and after supplementation with methionine.

Panels 1 and 2: CT and BALF images for Patients 1 (P1) and 3 (P3) respectively. A: CT at diagnosis. B: CT at the last follow-up. C-F: Pathological aspects of BALF. C and E: first WLL. D and F: last BAL. C-D: Periodic Acid Schiff staining (PAS), 100x. E-F: Oil Red O (ORO) staining, 200x.

Panel 1. A: The CT, that was performed very early in the course of the disease, showed discrete lesions with anterior hyperinflation, low-density ground-glass opacities, intralobular lines, and thickened interlobular septa with an anteroposterior density gradient. B: After 13 months of treatment the CT showed a disappearance of the crazy paving pattern. C-D: PAS staining showed the complete disappearance of the abnormal extra-cellular lipoproteinaceous material, highly present before treatment, after three months of supplementation. E-F: This was

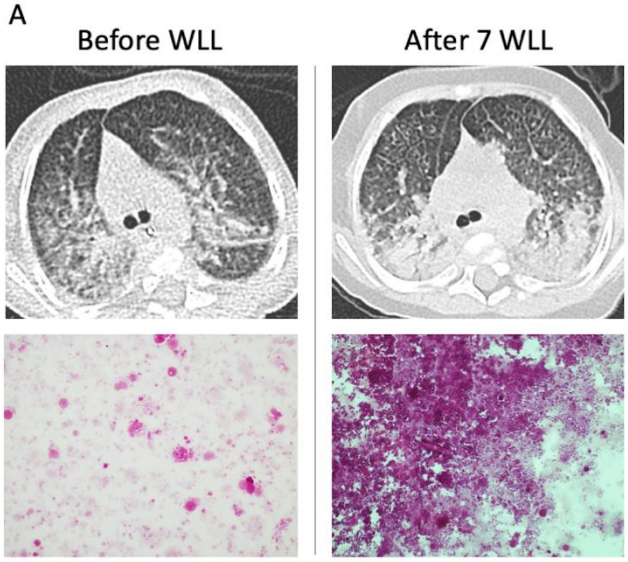
associated with a decrease in total cellularity of the fluid and in the number of ORO-positive macrophages, ranging from 100% before to 13% under treatment at D90.

Panel 2: A: The CT showed diffuse ground-glass opacities, thickened interlobular septa and intralobular lines (crazy paving pattern), with an increasing gradient of density in posterior and inferior areas. B: After 11 months of treatment the CT showed a clear improvement, with complete regression of the subpleural pseudonodular consolidations and of the crazy paving pattern. A subtle crazy-paving pattern of very low-density remained. C-D: PAS staining showed partial regression of the abnormal extra-cellular lipoproteinaceous material, highly present before treatment, after five months of supplementation. E-F: This was associated with a decrease in cellularity of the fluid and in the number of ORO-positive macrophages ranging from 90% before to 7% after 5 months of treatment.

Panel 3, Patient 2 (P2): A: Chest CT before starting methionine but after 25 WLL, several series of methylprednisolone pulses and 15 months of treatment with mycophenolate mofetil, showed no specific lesion apart from discrete ground glass opacities that could be related to breathing movements. B: chest CT performed after 18 months of supplementation with methionine and after 6 months of MMF arrest showed no specific lesion and especially no sign of fibrosis.

Panel 4, Patient 4 (P4): A: Chest CT before starting methionine but after 19 WLL and 4 series of methylprednisolone pulses, showed a crazy-paving appearance with an increasing gradient of density in the posterior and inferior areas, along with sub-pleural and intra-parenchymal microcystic lesions. B: A chest CT performed after 12 months of supplementation with methionine showed an important regression of consolidations. Microcystic lesions remained stable. Met: methionine. Suppl.: supplementation.

A


Before WLL
After 7 WLL

B

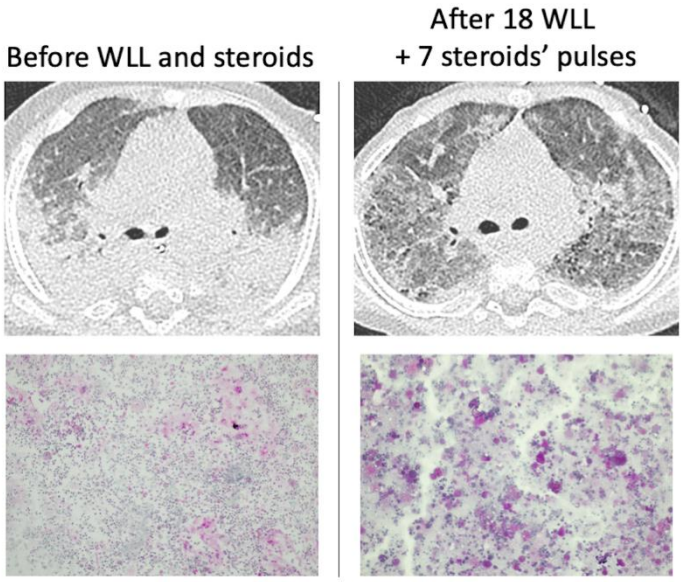

Before WLL and steroids
After 18 WLL
+ 7 steroids' pulses

Figure 2. Chest CT and pathological images for historical controls.

A: Chest CT images and PAS staining of BALF from a five-month-old boy at diagnosis (left panel) and six months later (right panel) after 9 WLL. Chest CT and BAL images show worsening of consolidations and an increasing amount of PAS positive material over time, respectively. B: Chest CT images and PAS staining of BALF for a three-month-old boy at diagnosis (left panel) and 12 months later (right panel) after 18 WLL and seven series of IV steroid pulses. Chest CT images show the persistence of the crazy-paving pattern, with partial regression of consolidations and the appearance of signs of fibrosis with microcystic lesions. BAL cytological analyses show the persistence of abundant extracellular and intra-macrophage PAS positive material. WLL: whole lung lavages.

Methionine supplementation for multiorgan dysfunction in MetRS-related pulmonary alveolar proteinosis

Online Supplement

METHODS

ADMINISTRATION SCHEME AND TARGETED RANGES

The frequency of medication was based on the known half-life of the molecule [1] and the peak was determined by performing kinetic measurements on the patients during the first day of supplementation. The initial dose was determined based on the usual mean methionine intake in alimentation for infants and children in France (available at https://www.anses.fr/fr/system/files/NUT-Ra-Proteines.pdf), with the initial aim to double methionine intake. The targeted plasma concentrations were defined according to available published data on normal methionine concentrations in children and on congenital disorders leading to hypermethioninemia and its potential toxicity. The normal fasting concentration should not exceed 45 µM [2]. Congenital hypermethioninemia is described notably in patients with methionine adenosyltransferase I/III (MAT I/III) or cystathionine beta-synthase deficiency. The consequences of high blood levels of methionine are liver dysfunction and central nervous system (CNS) abnormalities, especially with a risk of cerebral oedema. In a large series of patients with MAT I/III deficiency, CNS abnormalities were observed for those with mean plasma methionine values > 800 µM, whereas those with mean plasma methionine values < 800 µM generally did not have such abnormalities [3]. No instances of hepatic malfunction were detected in the aforementioned series. The authors recommended considering a dietary methionine restriction when plasma levels exceeded 500 µM and to only clinically monitor the patients with levels below 500 µM [3]. We thus decided to target methionine plasma

levels between 45 and 500 µM to obtain levels above the normal range but below the toxic range.

WESTERN BLOT

Neutrophils were isolated from the blood of Patient 1 and a control as described previously [4] After hypotonic lysis of erythrocytes, the neutrophil pellets were collected and washed in PBS. Neutrophils (10^7 cells in 500 µl HBSS) were then incubated with the proteinase inhibitor DFP (2.5 mM), followed by lysis with 125 µl concentrated modified Laemmli sample buffer (5X) containing 50 µg/mL pepstatin, 50 µg/mL leupeptin, 25 mM NaF, 12.5 mM Na3VO4, 12.5 mM EDTA, 12.5 mM EGTA, 6.25 mM p-NPP, and 50 µg/mL aprotinin.[5] Samples were denatured in boiling water (100°C) for 3 min and stored at −80°C until use. Samples were thawed and sonicated for 10 s before use and then subjected to classical 10% SDS–PAGE[5]. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in a mixture of tris-buffered saline and Tween-20. The membranes were then incubated overnight at 4°C in a solution containing a specific anti-MARS antibody (Abnova H00004141-B01P), followed by incubation in secondary antibodies (Santa Cruz, Heidelberg, Germany). Blots were visualized using ECL Western blotting reagents (Amersham Pharmacia).

PRIMING OF ROS PRODUCTION BY PERIPHEAL PHAGOCYTES/MONOCYTES

Whole blood from Patients 1 and 3 and a control was collected from lithium heparinized tubes (500 µl) and incubated for 15 min at 37°C with dihydrorhodamine 123(DHE) (Sigma-Aldrich). Samples were then treated for 1 h at 37°C with GM-CSF (10 ng/ml; R&D Systems), followed by stimulation for 5 min with fMLF (10-5M; Sigma-Aldrich). The reaction was stopped by adding 1 ml ice-cold lysis solution (BD Biosciences) and incubating for 5 min on ice. Samples were then washed with PBS (Sigma-Aldrich) and the pellets resuspended in 300 µl FacsFlow

solution (BD Biosciences). Samples were analysed by flow cytometry on a FACSCanto II (BD Biosciences). Phagocytes (neutrophils and monocytes) were selected on an FSC-SSC dot plot. Events (50,000) were recorded at a constant PMT voltage. Results are expressed as the index of stimulation (MIF DHE GM-CSF + fMLP/DHE GM-CSF alone).

RESULTS

METHIONINE ADMINISTRATION

Methionine plasma concentrations were measured to adapt the doses of the supplementation. Twenty-four-hour kinetic studies were performed on days (D) 1 and 3 of treatment and at least one time later before M2. After that, methionine plasma concentrations were regularly monitored at residual and peak states for one or two intakes. Within each patient, treatment every 6h led to reproducible residual and peak values for 24-h periods throughout days and months of treatment (Supplemental Figure S1).

WESTERN BLOT

MetRS protein levels in PBMCs of P1 before starting methionine were normal relative to those of a control individual (Supplemental Figure S3).

PRIMING OF ROS PRODUCTION BY PERIPHEAL PHAGOCYTES/MONOCYTES

We assessed ROS production by peripheral monocytes of P1 and P3 before and after three months of treatment. Before treatment, GM-CSF priming of ROS production by peripheral monocytes (Supplemental Figure S3) stimulated by GM-CSF and fMLP was low (stimulation index relative to control of 46% for P1 and 58% for P3). After three months of treatment, the stimulation index relative to control normalized for P1 (109%) and improved for P3 (73%) (Supplemental Figure S3).

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FIGURE LEGENDS:

Supplemental Figure S1. Kinetic data of residual (solid lines) and peak (dotted lines) plasma methionine values.

The peak was determined during a kinetic study to be 1 h after taking the medication. A and B: Complete kinetic study measuring the residual and peak concentrations at each intake over 24 h. Data are shown for Patient 1 after one year of treatment (A) with a methionine dose of 110 mg/kg/day and for Patient 3 on day 3 of the treatment (B) with a methionine dose of 80 mg/kg/day. C and D: Residual and peak plasma values on three different days under the same dosage. Data are shown for Patient 2 after 10 (M10), 11 (M11), and 13 (M13) months of treatment with a methionine dose of 80 mg/kg/day and for Patient 4 on three different days (D5, D10, and D11) during the same month (M2) of treatment with a methionine dose of 90 mg/kg/day. Within each patient, giving treatment every 6 hours allowed getting reproducible residual and peak value during 24-hour periods and also across days and months of treatment.

Supplemental Figure S2. Comparison of clinical and biological parameters with historical controls.

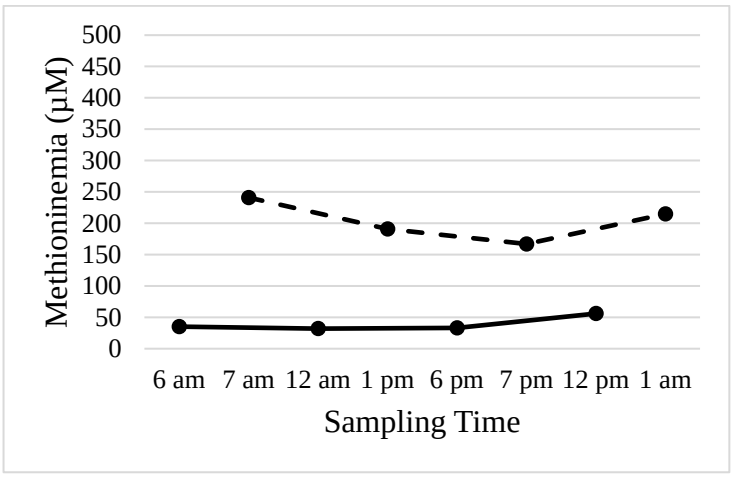
Comparison of the data between Patients 1, 3, and 4 (solid lines) and historical controls (median value and IQR, dotted line) are shown for SD of weight, respiratory rate (RR), haemoglobin (Hb), white blood cell (WBC), neutrophils, and platelets counts, CRP, albumin, prothrombin rate (PT), AST, GGT and total bilirubin (T Bilirubin). M0: D0 or diagnosis; M6-M12: last follow-up for treated patients and six months to one year of the progression of the disease for historical controls. The lower limit of the normal is designated by a horizontal dotted line for haemoglobin, albumin, and the prothrombin rate; the upper limit of normal is designated by a horizontal dotted line for WBC, neutrophils, and platelets counts, CRP, GGT, and total and conjugated bilirubin. Differences between values at M0 and M6-M12 were statistically

significant between historical controls and treated patients for respiratory rate (p=0.025), blood neutrophils (p=0.034), AST (p=0.004) and GGT (p=0.038).

Supplemental Figure S3. Cellular analyses for Patients 1 and 3.

A: Analysis of MetRS protein expression for Patient 1. The MetRS protein was normally expressed in PBMCs relative to those of a control individual. B: GM-CSF priming of ROS production by peripheral monocytes before (M0) and after three months of treatment (M3) for Patient 1 (P1) and Patient 3 (P3). Priming of ROS production by peripheral monocytes stimulated by GM-CSF and fMLP was measured in patients and controls and the stimulation index expressed as the percentage of the control values. The control value was thus considered to be 100%. The stimulation index relative to control for both patients before treatment was low: 46% for P1 and 58% for P3. After three months of treatment, the stimulation index relative to control normalized for P1 (109%) and improved for P3 (73%).

A



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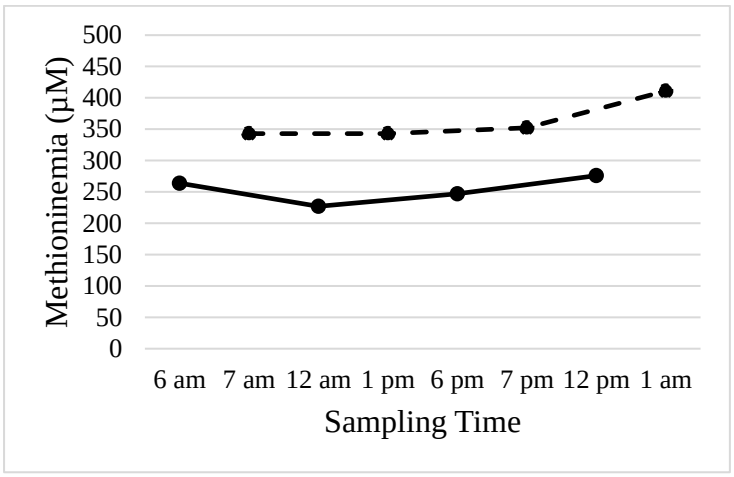

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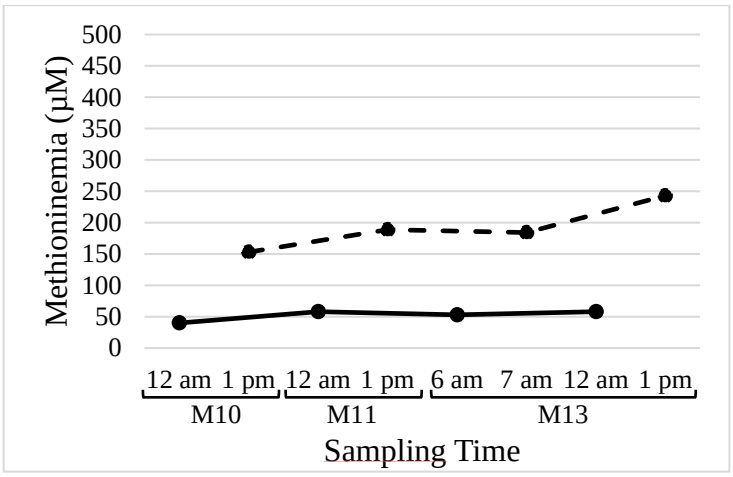

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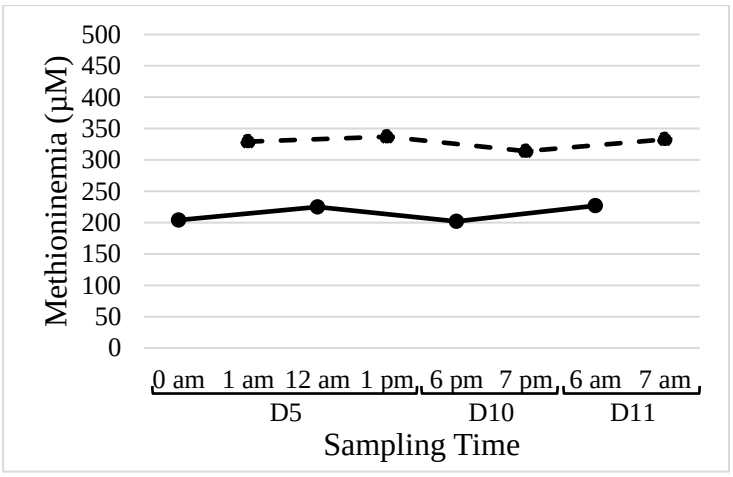
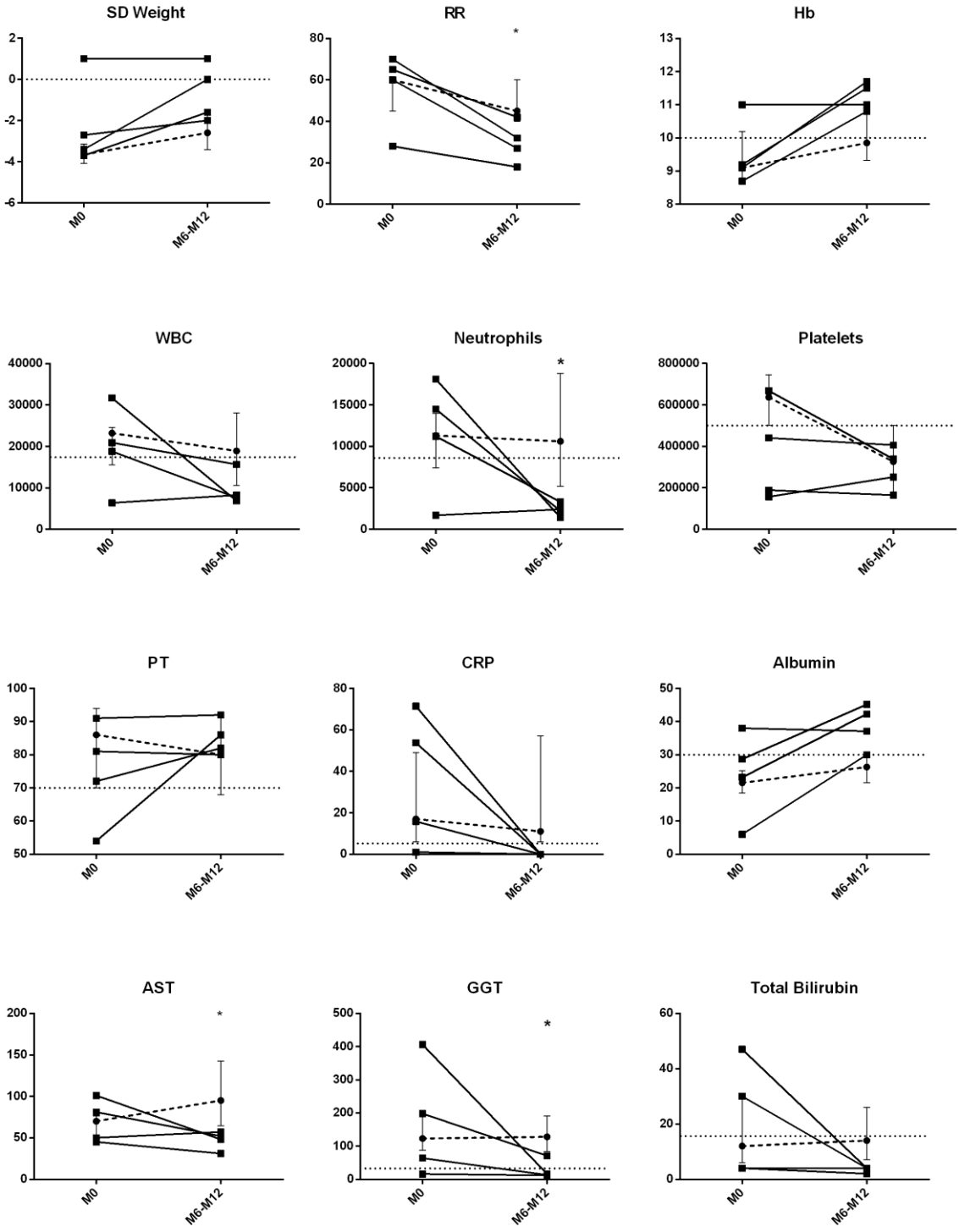
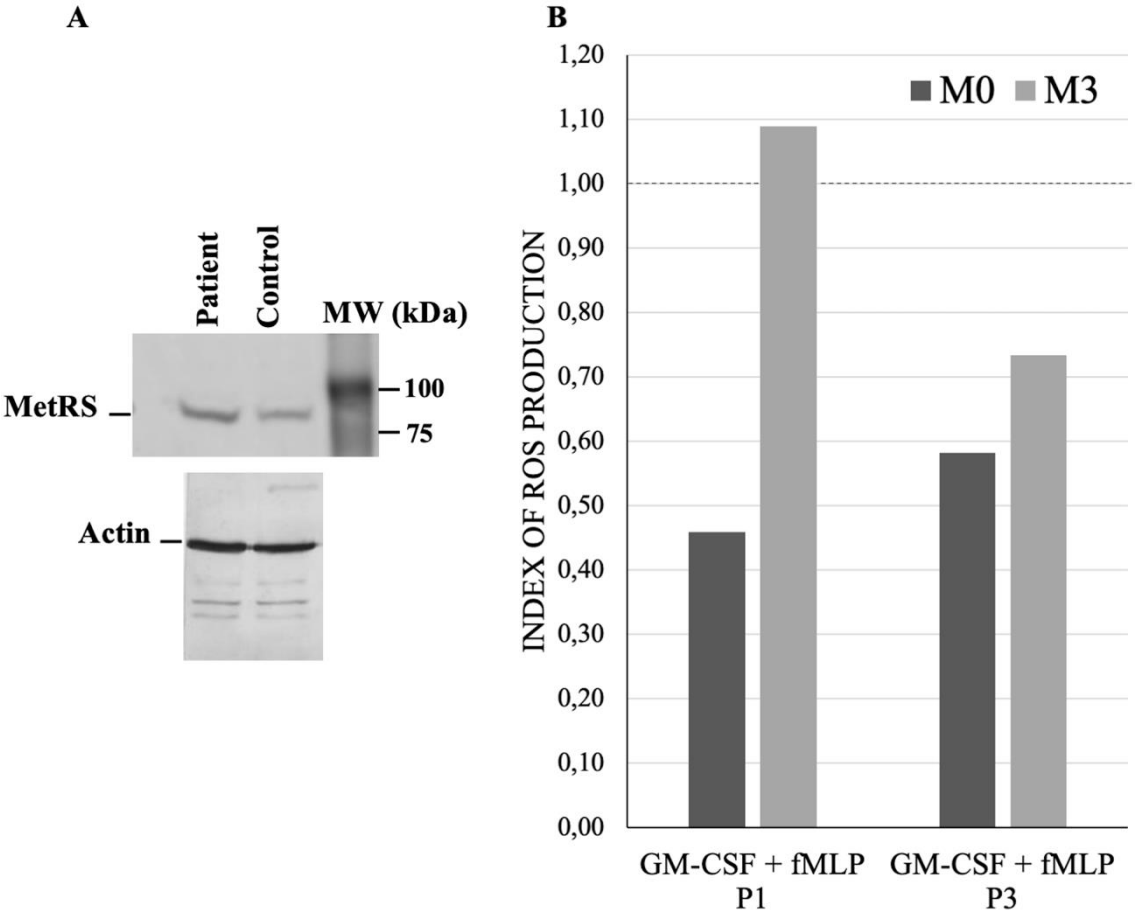


Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3